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Neurodegeneration Is Associated to Changes in Serum Insulin-like Growth Factors

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Serum levels of insulin and insulin-like growth factors and their binding proteins (IGFs and IGFBPs, respectively) are changed in human neurodegenerative diseases of very different etiology, such as Alzheimer's disease, amyotrophic lateral sclerosis, or cerebellar ataxia. However, the significance of these endocrine disturbances is not clear. We now report that in two very different inherited neurodegenerative conditions, ataxia-telangiectasia (AT) and Charcot-Marie-Tooth 1A (CMT-1A) disease, serum levels of IGFs are also altered. Both types of patients have increased serum IGF-I and IGFBP-2 levels, and decreased serum IGFBP-1 levels, while only AT patients have high serum insulin levels. Furthermore, serum IGFs are also changed in three different animal models of neurodegeneration: neurotoxin-induced motor discoordination, diabetic neuropathy, and hereditary cerebellar ataxia. In these three models, serum insulin levels are significantly decreased, serum IGF-I and IGFBP-1, -2, and -3 are decreased in diabetic and neurotoxin-injected rats, while serum IGFBP-1 is increased in hereditary ataxic rats. Altogether, these observations indicate that a great variety of neurodegenerative diseases show endocrine perturbations, resulting in changes in serum IGFs levels. These perturbations are disease-specific and are probably due to metabolic and endocrine derangements, nerve cell death, and sickness-related disturbances associated to the neurodegenerative process. Our observations strongly support the need to evaluate serum IGFs in other neurodegenerative conditions. © 2000 Academic Press

Key Words: **insulin-like growth factors; neurodegeneration; ataxia; diabetes; Charcot-Marie-Tooth disease.**

INTRODUCTION

Recent progress in human genetics have revealed the existence of specific mutations in many types of neurodegenerative diseases (Hardy and Gwinn-Hardy, 1998). Because the affected proteins are usually widely expressed in the nervous system, the relationship between changes in protein function and appearance of specific patterns of cell death is not yet well understood (Price *et al.*, 1998). Conceivably, the primary pathogenic effect will lead to death of those cells directly affected by the mutation. Subsequent homeostatic derangements associated to this primary cell death may include a loss of appropriate trophic input to nerve cells not primarily affected by the disease. As a consequence, secondary cell death will proceed. A similar general process is envisaged for nongenetic neurodegenerative processes such as those taking place after isquemic insult, physical injury, or neurotoxin-related neuronal death. Thus, changes in growth factor input, among other pathological alterations, may be an additional factor involved in the progression of neurodegenerative processes. In this regard, we and others previously reported altered levels of

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insulin, insulin-like growth factor I (IGF-I), and IGF-binding proteins (IGFBPs) in different human neurodegenerative diseases (Tham *et al.*, 1993; Torres-Aleman *et al.*, 1996, 1998; Schwab *et al.*, 1997; Craft *et al.*, 1998). More recently, we also found that insulin-like growth factor I (IGF-I) is involved in the progression of neuronal death after neurotoxin insult in rats (Fernandez *et al.*, 1999).

The insulin family of growth factors include insulin, IGF-I and -II, and the IGFBPs. The latter are proteins that modulate the biological activity of the IGFs (Jones and Clemmons, 1995). These peptides are found at high levels in the blood stream, but are also present in the developing and adult nervous system where they exert wide trophic actions (Torres-Aleman, 1999). IGF-I has recently been found of potential therapeutical use in different neurodegenerative conditions (Fernandez *et al.*, 1998; Pulford *et al.*, 1999). The rationale for these studies is based on the potent trophic actions of IGF-I and on its ability to rescue injured neurons after a great diversity of insults (Dore *et al.*, 1997; Torres-Aleman and Fernandez, 1998). The observation of low serum IGF-I levels in human patients suffering from different types of neurodegenerative diseases and in animal models (Torres-Aleman *et al.*, 1996; Busiguina *et al.*, 1996; Schwab *et al.*, 1997; Scheepens *et al.*, 1999) gave support to this therapeutic approach. However, it is still unclear whether insulin (Wickelgren, 1998), IGF-I (Torres-Aleman *et al.*, 1996, 1998; Dore *et al.*, 1997), or for that matter any neurotrophic factor, are directly involved in the progression of the neurodegenerative process. In the present study we have evaluated widely different types of etiopathogenic processess involved in neurodegeneration to determine whether changes in serum levels of these growth factors are characteristic of a subset of neurodegenerative diseases or, on the contrary, they changed in all types of neurodegenerative conditions regardless of their origin. We include both human diseases and animal models in our study to have a broader sample of neurodegenerative mechanisms. Specifically, we have explored whether both acute (neurotoxic insult) or slow (metabolic derangement) nongenetic neurodegenerative processes as well as hereditary neurodegenerative diseases (ataxia-telangiectasia, Charcot-Marie-Tooth 1A disease, and cerebellar atrophy) will present changes in these trophic factors. We found significant changes in serum insulin, IGF-I, and IGFBPs levels in all these neurodegenerative conditions of widely different phenotypes or genotypes.

MATERIALS AND METHODS

Patients

The series comprises 29 patients with an inherited peripheral neuropathy of the Charcot-Marie-Tooth 1A (CMT-1A) disease type. Most of these patients belong to six previously reported families (Hallan *et al.*, 1992; Garcia *et al.*, 1998). Mean age was 33.4 years, with a range of 8–77 years. All patients showed slight to moderate signs of peroneal muscular atrophy, marked and uniform slowing of nerve conduction velocity, and a tandem duplication of a 1.5-Mb region in chromosome 17p11.2. The weight/height ratio of CMT-1A patients was within normal population parameters. A second series of 12 patients with an inherited central neurodegeneration, ataxia-telangiectasia (AT), were also studied (13.2 years; range, 3–34). They were genotyped, diagnosed, and their blood samples collected through a multicenter program organized by the AT Children's Project (Boca Raton, FL). Age-matched normal subjects (age range of 6–68 years) were used as controls: 10 for CMT-1A (34.4 years) and 8 for AT (12.2 years). Since the two populations of control subjects showed similar values for serum IGF-I and insulin they were pooled and shown as a single control population. Blood samples were withdrawn in fasted conditions between 8 and 10:00 a.m.

Animal Models

Neurotoxin-induced cerebellar ataxia. Cerebellar deafferentation resulting in ataxia was induced in adult rats (250–300 g) by injection of the neurotoxin 3-acetylpyridine (3AP, 50 mg/kg ip) as described in detail before (Fernandez *et al.*, 1997). Massive neuronal loss (99%, $P < 0.001$) was found specifically in the inferior olive (Torres-Aleman *et al.*, 1991). Animals show permanent and severe ataxia as measured in the rota-rod test (Fernandez *et al.*, 1998). They also had a 10% decrease in body weight as compared to controls.

Hereditary cerebellar ataxia. Two to three month old "shaker" mutant rats with hereditary ataxia due to progressive primary Purkinje cell death were used (Tolbert *et al.*, 1995). At these ages most Purkinje cells in the cerebellar anterior lobe and many of these cells in the posterior lobe have degenerated. Coincident with this loss of Purkinje cells the mutant rats are ataxic characterized by a wide-based "staggering" gait. Ataxic animals weighed 20–28% less than age-matched controls (Wolf *et al.*, 1996).

Diabetic neuropathy. Insulin-dependent diabetes was induced in adult rats by injection of the pancreatic beta-cell toxin streptozotocin (65 mg/kg, ip) as described (Busiguina *et al.*, 1996). Diabetic rats had significantly high serum glucose levels (396 ± 16 mg/dl as compared to 72 ± 2 mg/dl in controls) and developed a peripheral neuropathy as determined by loss in the paws of the sensitivity to heat ($P < 0.05$ as compared to controls). Central-neuronal loss (Biessels *et al.*, 1994) was ascertained by counting cerebellar Purkinje cells; we found a 25% reduction in the number of this type of neurons ($P < 0.05$) after 2 months of induction of insulin-dependent diabetes. Animals show also a severe body mass loss (50% of controls).

Immunoassays

All assays used have been described in detail before (Busiguina *et al.*, 1996; Torres-Aleman *et al.*, 1996, 1998). Two types of samples were analyzed: serum from human subjects and from experimental animals; and cerebellar tissue from experimental animals. We chose the latter as a representative brain area because previous findings indicated that the cerebellum shows specific changes in the levels of insulin-like growth factors in neurodegenerative diseases (Torres-Aleman, 1991, 1996). IGF-I, IGFBPs, and insulin levels were measured by either radioimmunoassay (IGF-I, insulin, IGFBP-2, and BP-3), ELISA (BP-1), or Western ligand blot (WLB). The latter technique was used to measure intact BP-3 in human sera as determined by its affinity to bind labeled IGF-I. Immunoreactive BP-3 levels in human serum do not reflect intact levels of BP-3 because most anti-human anti-BP-3 antibodies recognize BP-3 fragments (Marks *et al.*, 1991). WLB was used also to measure rat IGFBPs in serum and brain tissue. In all cases, control and experimental samples were alternated in the blot to minimize intrablot variations. WLB results were analyzed by laser densitometry and results express as percentage of control levels within the same blot (see figures).

Serum samples were either directly measured (IGFBPs and insulin) or extracted (for IGF-I) using Sep-pak cartridges as reported (Pons and Torres-Aleman, 1992). Brain tissue samples were processed for IGF-I RIA or BP-2 WLB as described (Pons and Torres-Aleman, 1992; Busiguina *et al.*, 1996). Results are shown as mean SEM. A Student's *t* test was used for statistical analysis. A value of $P < 0.05$ was taken as significantly different.

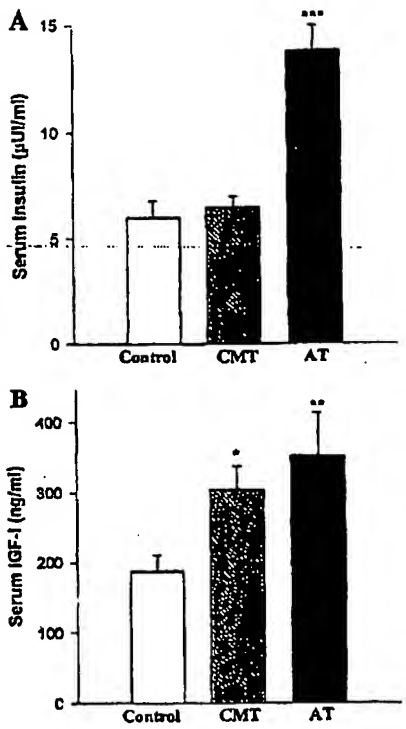

FIG. 1. Levels of insulin-like growth factors in human inherited neurodegeneration. Serum insulin (A) and IGF-I (B) levels in Charcot-Marie-Tooth 1A disease (CMT) patients, ataxia-telangiectasia (AT) patients, and control subjects. $^{*}P < 0.05$; $^{**}P < 0.005$; $^{***}P < 0.0001$.

RESULTS

Serum Insulin-like Growth Factors in Hereditary Neurodegenerative Diseases

We previously reported that in genetically heterogenous cerebellar ataxic patients IGF-I and insulin levels are significantly depressed (Torres-Aleman *et al.*, 1996). We now studied two human neurodegenerative conditions where the genetic perturbation is homogenous and identified. Ataxia-telangiectasia (AT) is associated to a mutation in Atm, a protein belonging to the superfamily of PI3-kinases (Savitsky *et al.*, 1995), while Charcot-Marie-Tooth type 1A disease (CMT-1A) is linked to a duplicaton of the gene for PMP22, a constitutive myelin protein (Hanemann and Muller, 1998). As shown in Fig. 1, serum IGF-I levels were significantly increased in both types of patients while

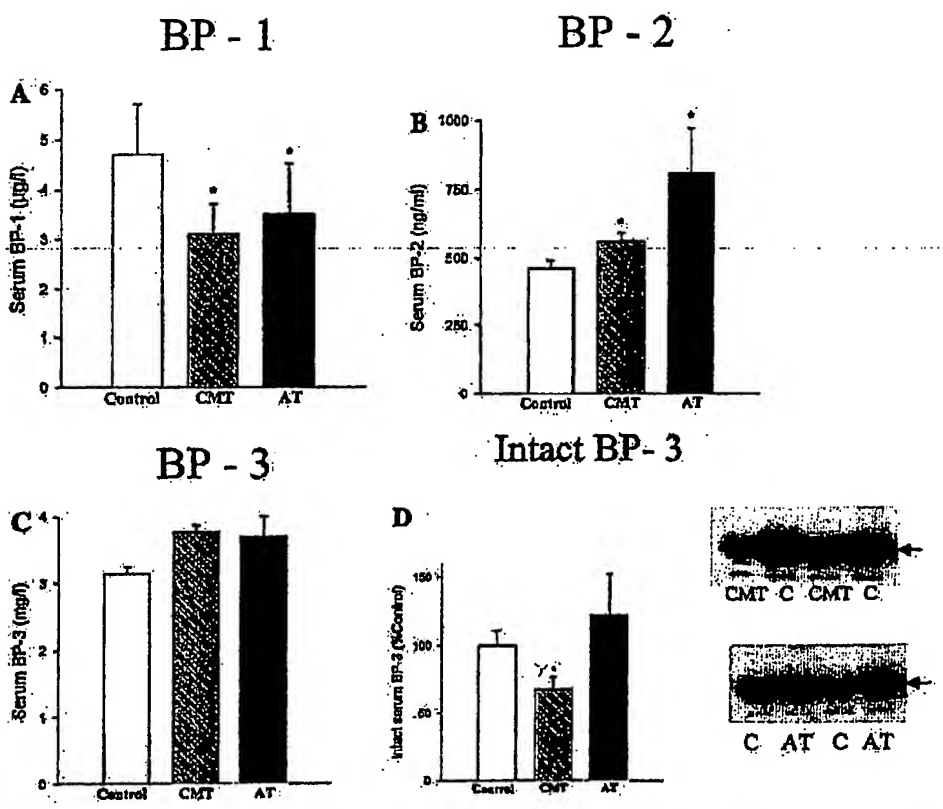

FIG. 2. Serum levels of BP-1 (A), BP-2 (B), and BP-3 (C) in CMT and AT patients and in controls (c). Intact BP-3 (D) determined by its affinity to bind IGF-I (WLB) was significantly changed in CMT patients. Representative WLBs of BP-3 in CMT and AT patients and controls are shown to the right in D. $^*P < 0.05$.

serum insulin levels were significantly increased only in A-T patients. Similarly, serum levels of 2 of the 3 major types of circulating IGFBPs were also altered (Fig. 2). Although immunoreactive BP-3 levels were not changed (Fig. 2C), bioactive levels, as determined by the affinity of BP-3 to bind IGF-I, were slightly increased in AT patients (122 ± 35% of control values) and significantly decreased in CMT patients (67 ± 9% of controls, $P < 0.05$; Fig. 2D).

We then extended these observations to an animal model of cerebellar ataxia (the "shaker" rat) due to X-linked inheritance of the shaker genotype which results in the adult-onset degeneration of Purkinje cells. These rats have low serum insulin levels, and although they also have very low cerebellar IGF-I levels, serum IGF-I levels are normal (Fig. 3). In addition, serum and cerebellar IGFBPs are also modified in these animals (Fig. 3).

Serum Insulin-like Growth Factors in Nongenetic Neurodegeneration

Altered serum insulin and IGF-I levels have also been found in human and experimental neurodegenerative conditions not linked to genetic mutations (Tham *et al.*, 1993; Schwab *et al.*, 1997; Craft *et al.*, 1998; Fernandez *et al.*, 1998; Scheepens *et al.*, 1999). We now have extended these observations in two different models of experimental neurodegeneration. A first one consists of an acute neurodegenerative process due to toxic insult (3AP). In this model, neuronal death is circumscribed to the first week after neuro-

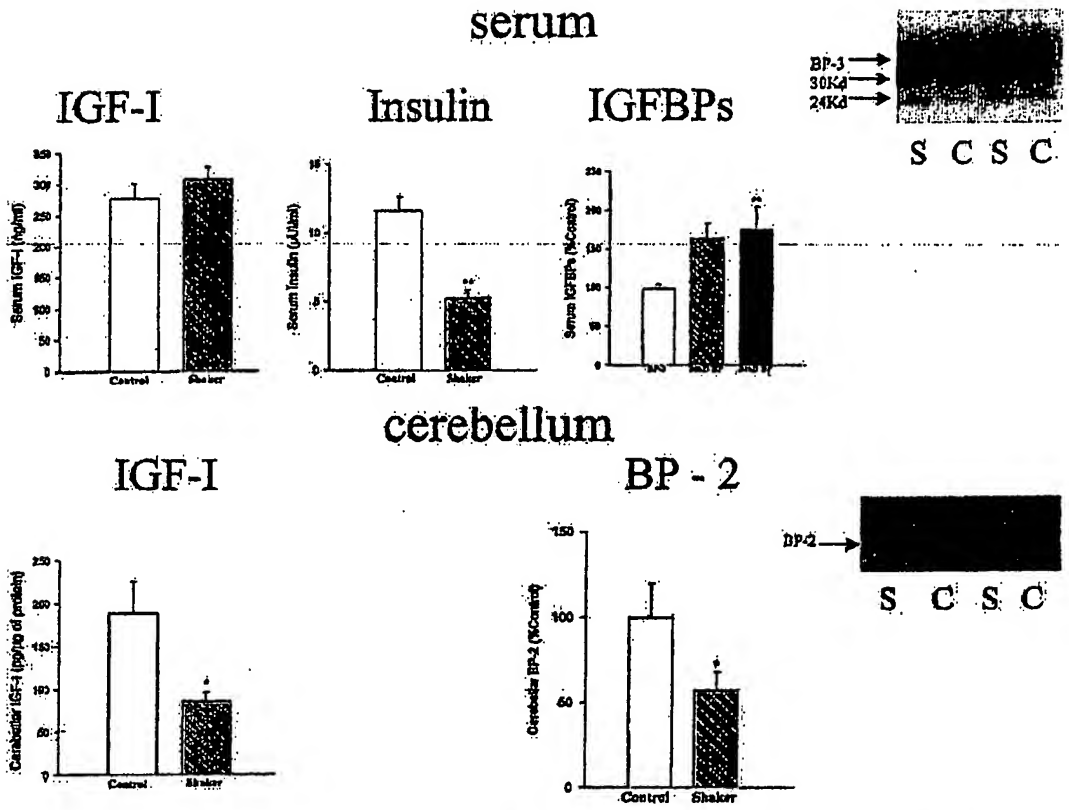

FIG. 3. Insulin-like growth factors in an animal model of hereditary cerebellar ataxia (shaker rat). Upper panel: serum levels of insulin, IGF-I, and IGFBPs in shaker ataxic rats. Lower panel: cerebellar levels of IGF-I and BP-2 in shaker rats. Upper inset shows a representative WLB for serum IGFBPs while the lower inset shows a representative WLB for BP-2 in the cerebellum. S, shaker rat; C, control rat. Arrows in gels indicate position of the different IGFBPs. $^{*}P < 0.05$; $^{**}P < 0.001$.

toxin administration (Fernandez *et al.*, 1999). A second one is a prolonged neurodegenerative process due to diabetes-related metabolic derangements, where cell death develops gradually and continues unabated. As previously reported (Busiguina *et al.*, 1996; Fernandez *et al.*, 1998), both types of insults produce low serum IGF-I levels. However, time-course analysis of the changes indicate that in diabetic animals serum IGF-I remains low for the duration of the study (8 weeks), while 3AP-injected rats show a recovery of serum IGF-I 4 weeks after injection of the neurotoxin (Fig. 4A). If the components of the IGF trophic system are evaluated at a time when serum IGF-I levels are depressed, i.e., 2 weeks after 3AP and 8 weeks after streptozotocin, most of them are also altered. Thus, as shown in Fig. 4B, serum insulin is significantly decreased. Similarly, a pronounced decrease in cerebellar IGF-I levels is also found (Fig. 4C). Finally, both serum and cerebellar IGFBPs are also low (Figs. 4D and 4E). However, if these components are evaluated 4 weeks after 3AP injection, when IGF-I levels are back to normal, all of them are also normalized (not shown).

DISCUSSION

The present findings extend and reinforce the observation that levels of circulating insulin, IGF-I and IGFBPs are altered in many types of human neurode-

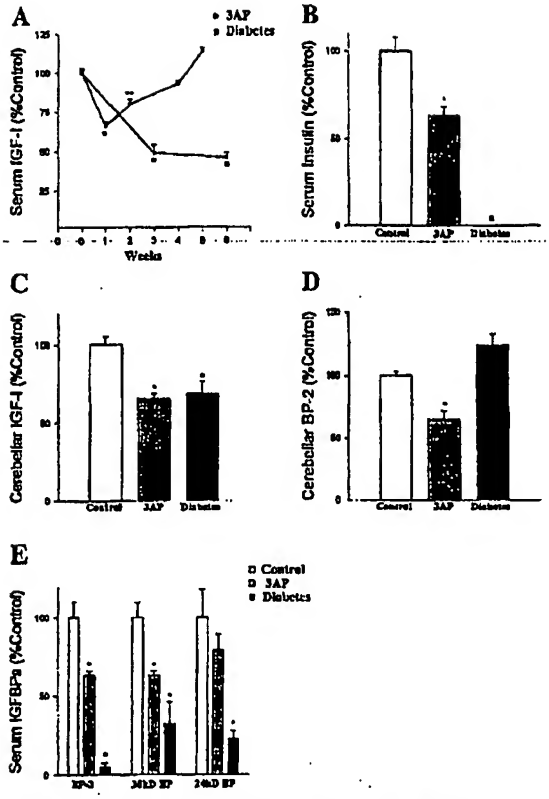

FIG. 4. (A) Time course analysis of serum IGF-I levels in acute, neurotoxin-induced (3AP) neurodegeneration, and in long-term neurodegeneration (diabetes). (B) Serum levels of insulin 2 weeks (3AP) and 8 weeks (diabetes) after toxin administration. (C) Cerebellar levels of IGF-I in ataxic (3AP) and diabetic animals. (D) Cerebellar levels of BP-2 in 3AP-treated and diabetic animals. (E) Serum IGFBPs in 3AP-induced ataxic rats (3AP) and in insulin-dependent diabetic rats. a, undetectable levels. $^{*}P < 0.01$; $^{**}P < 0.05$.

generative diseases, including major illnesses such as Alzheimer's disease or stroke as well as inherited neurodegenerative diseases (Tham *et al.*, 1993; Torres-Aleman *et al.*, 1996, 1998; Schwab *et al.*, 1997; Craft *et al.*, 1998). Similarly, changes in serum insulin, IGF-I and IGFBPs are found in various animal models of neurodegeneration (Torres-Aleman *et al.*, 1991; Fernandez *et al.*, 1998; Zhang *et al.*, 1997, 1999). Although experimental models do not mimic all aspects of human disease, altogether this suggests that alterations in insulin/IGF-I input may be common to many, if not all neurodegenerative diseases.

It is well known that changes in growth factor and cytokine levels, including the IGFs and the IGFBPs (Torres-Aleman and Fernandez, 1998) take place at the lesioned site in all types of neurodegenerative processes, both in humans and in animal models (Isackson, 1995; Toulmond *et al.*, 1996; Feldman *et al.*, 1997; Raivich *et al.*, 1999). These changes, in most instances resulting in increased local levels of the growth factors, are considered to reflect local adaptive responses to cell death. However, it is intriguing that serum levels of insulin growth factors are changed not only in peripheral neurodegenerative diseases but even in degeneration affecting central neurons. In this regard, it has been reported that exogenously administered insulin and IGF-I can access the brain (Pardridge, 1993; Poduslo *et al.*, 1994; Reinhardt and Bondy, 1994; Carro *et al.*, 2000). While the physiological significance of these observations is currently under intense scrutiny (Aberg *et al.*, 2000; Carro *et al.*, 2000), recent findings suggest that passage of serum insulin and IGF-I through the blood–nerve barriers may be of pathological importance (Armstrong *et al.*, 2000). Furthermore, while the role of endogenous IGFs in the brain is still not clear, administration of IGFs results in potent neuroprotective effects in a great variety of experimental conditions (Feldman *et al.*, 1997; Fernandez *et al.*, 1998; Pulford *et al.*, 1999). Thus, it is conceivable that IGF therapy of neurodegenerative diseases could be implemented through peripheral administration.

It is intriguing that the pattern of changes in IGF-I, and IGFBPs in AT and CMT patients is identical (although insulin is increased only in AT patients) because these diseases are very different both in phenotype and in genotype. Similarly, animal models as diverse as diabetic rats, 3AP-ataxic rats or pcd ataxic mice (Zhang *et al.*, 1997, 1999) show identical changes in insulin, IGF-I, and IGFBPs (although insulin levels in pcd mice have not been reported). Thus, and independently of the etiology of the disease, we can distinguish two general situations: (1) Deficiency states: diseases with low insulin and/or IGF-I levels; and (2) Resistance states: diseases with high insulin and/or IGF-I levels. For the sake of clarity we do not take into account changes in IGFBPs levels because they are usually considered to be secondary to changes in insulin/IGF-I levels (Ferry *et al.*, 1999).

Deficiency states such as those found in cerebellar ataxia, amyotrophic lateral sclerosis, or stroke (Torres-Aleman *et al.*, 1996, 1998; Schwab *et al.*, 1997) may arise from a variety of causes that will ultimately lead to impaired hormone production due to either death of the hormone-synthesizing cells, cell stress, or endo-

crine disregulation. While death of pancreatic beta cells producing insulin or of hepatocytes producing IGFs is unlikely, except in neurodegeneration associated to severe endocrine diseases, our results suggests that endocrine alterations, resulting in low hormone levels may be a problem far more commonly associated to neurodegeneration than previously thought.

High IGF-I and insulin levels found in AT, CMT, and Alzheimer's disease (Tham *et al.*, 1993; Craft *et al.*, 1998) reflect a resistant state and are likely due to a loss of sensitivity of target cells to the actions of the growth factor. Although resistance may occur with low or even normal growth factor levels, high circulating levels of either insulin or IGF-I always reflect a resistant state (Jain *et al.*, 1998). Possible mechanisms leading to insulin/IGF-I resistance in neurodegenerative diseases may be varied. Genetic mutations associated to neurodegeneration may include proteins putatively involved in the insulin/IGF signalling pathway. This might be the case of the Atm protein mutated in AT patients which is a PI 3-kinase family member. PI 3-kinases are critically involved in the biological actions of IGFs and insulin (LeRoith *et al.*, 1995). Another potential interaction with the IGF pathway is the dentatorubral-pallidoluysian atrophy protein, which interacts with the insulin receptor substrate (IRS, Okamura *et al.*, 1999), which is a pivotal molecular intermediary of insulin/IGF actions (LeRoith *et al.*, 1995). Other potential causes of resistance to insulin/IGF-I may be related to pathological changes in affected cells or in their vicinity, including altered cytokine production or glucose metabolism (Jain *et al.*, 1998; Venters *et al.*, 1999). The latter processes may be involved in cell death in Alzheimer's disease since both serum insulin and IGF-I levels are elevated (Tham *et al.*, 1993; Craft *et al.*, 1998). Furthermore, it has been suggested that high serum insulin levels in Alzheimer's disease reflects an altered insulin metabolism of affected neurons (Craft *et al.*, 1998; Wickelgren, 1998). Hence, a "neuronal diabetes-like" process could be primarily involved in the development of Alzheimer's disease (Wickelgren, 1998). We can speculate that similar "resistant states" of affected nerve cells underlie high insulin/IGF levels found in AT or CMT patients. If our hypothesis is correct, patients with dentatorubral-pallidoluysian atrophy may have high serum insulin/IGF-I levels.

Although we cannot yet determine the precise mechanisms leading to either increased or decreased serum levels of IGFs in neurodegenerative diseases, at least three types of general processes can be envisaged to participate. A first one may be signals associated to the cell death process, such as cytokines (Fan *et al.*, 1998) and other cellular mediators involved in neurodegeneration. This is supported by our observation that cell death is temporally correlated with serum IGF-I levels in models of acute and of progressive neurodegeneration (see also Zhang *et al.*, 1999). The gradual normalization of serum IGF-I levels after 3AP insult is not due to transient toxic effects of the drug since lesioning of inferior olive neurons by a different method elicits a similar decrease in IGF-I levels (Fernandez *et al.*, 1998). A second possible mechanism may be metabolic derangements associated to the neurodegenerative process. For example, a high prevalence of diabetes in Friedreich's ataxia, multiple sclerosis, or Wolfram syndrome has been reported (Wertman *et al.*, 1992; Barrett *et al.*, 1995; Ristow *et al.*, 1998). As already mentioned, central derangements in insulin action could be involved in Alzheimer's disease (Craft *et al.*, 1998; Wickelgren, 1998) and may also be involved in ataxia-telangiectasia (Knittweis, 1998). In this regard, it is important to note that many endocrine diseases are associated to nerve cell death. A third possibility is that changes in insulin/IGFs levels are secondary to general illness-related changes such as alterations in food comsuption, in the sleep–wake cycle, or in physical activity. Low protein intake, sleep disorders, critical illness, and intense physical activity have all been related to changes in the insulin/IGF axis (Jones and Clemmons, 1995; Frost and Lang, 1998; Simon 1998). The relative contribution of each of these processes would be specific for each disease, resulting in a distinct pattern of changes of insulin/IGF-I in each disease.

In summary, our results indicate that changes in serum insulin, IGF-I, and/or IGF-binding proteins are associated to many different types of neurodegenerative processes. These changes are probably due to a variety of endocrine alterations associated to the disease state. However, it is tempting to speculate that in a subset of neurodegenerative diseases these changes may directly reflect pathogenic mechanisms. At any rate, our results strongly support the need to evaluate serum IGFs and possibly other circulating growth factors in all types of neurodegenerative conditions. If circulating trophic factors are found to be altered in other diseases we may start considering that endocrine/metabolic alterations are commonly associated to neurodegenerative diseases. The recent success of subcutaneous IGF-I treatment in experimental models of neurodegeneration support the notion that interventions aimed to correct these endocrine alterations may lead to new therapeutic approaches.

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